

Preliminary assessment of the genetic diversity of Ecuadorian *Ilex guayusa* using *inter simple sequence repeat* (ISSR) markers

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Estudio preliminar de la diversidad genética de *Ilex guayusa* ecuatoriana mediante marcadores *inter simple sequence repeat* (ISSR)

Resumen

Evalúamos el grado de diversidad genética de *Ilex guayusa*, una especie de importancia etnobotánica y comercial para las comunidades indígenas de la Amazonía ecuatoriana. Caracterizamos genéticamente a 157 individuos, provenientes de chacras ubicadas a lo largo de seis provincias, con nueve marcadores ISSR (Inter Simple Sequence Repeats). Se detectó en total 91 bandas polimórficas, pero el índice de heterocigosidad estimada ($H_e = 0.19$) de la población reveló un nivel reducido de variabilidad genética para la especie. Los índices de distancias genéticas de Nei ($0.013 \leq D_s \leq 0.086$) revelaron un nivel reducido de divergencia genética entre individuos provenientes de provincias diferentes. De manera similar, el análisis de varianza molecular (AMOVA) demostró que solamente el 18% de la variación observada para *I. guayusa* ocurre entre poblaciones. El bajo grado de diversidad genética encontrado para *I. guayusa* puede ser atribuido al hecho de que esta especie es cultivada exclusivamente mediante propagación clonal; una actividad cultural que probablemente ha homogenizado el acervo genético de la especie a lo largo de su rango geográfico de cultivo. El análisis de coordenadas principales (PCoA) demostró que el germoplasma colectado puede estructurarse en tres grupos distintos caracterizados por un leve contraste genético en gradiente direccional, de norte a sur. La inclusión de un mayor número de muestras de provincias sub-representadas (ej. Sucumbíos) y poblaciones silvestres, si existen, ayudaría a esclarecer el nivel de diversidad genética y estructura poblacional de la especie, y la historia de su cultivo en la región.

Palabras Clave. Aquifoliaceae, Guayusa, propagación clonal, baja diversidad genética.

Abstract

We assessed the degree of genetic diversity of *Ilex guayusa*, a species of ethnobotanic and commercial relevance for indigenous communities of the Ecuadorian Amazon. We characterized 157 individuals, from small cultivation sites across six provinces, using nine Inter Simple Sequence Repeat (ISSR) markers. A total of 91 polymorphic bands were detected in, but estimated heterozygosity ($H_e = 0.19$) revealed a reduced level of genetic variability. Pairwise-Nei genetic distance indices ($0.013 \leq D_s \leq 0.086$) implied a reduced level of genetic divergence between individuals from different provinces. Accordingly, partitioning of genetic diversity (AMOVA) indicated that only 18% of variation observed for *I. guayusa* occurred between populations. The low degree of genetic diversity found for *I. guayusa* could be ascribed to the fact that the species is exclusively cultivated via clonal propagation; a cultural activity which has probably homogenized the species' genetic pool across its geographic range of cultivation. However, PCoA analysis resolved collected germplasm into three distinct groups displaying a subtle genetic contrast in a directional gradient, from north to south. The inclusion of a higher number of samples from underrepresented provinces (i.e. Sucumbíos), and wild populations, if they exist, would help resolve knowledge gaps regarding the genetic diversity and population structure of the species and its cultivation history in the region.

Keywords. Aquifoliaceae, Guayusa, clonal propagation, low genetic diversity.

INTRODUCTION

Ilex guayusa Loes. (Aquifoliaceae) is a tree species native to the Amazonian rainforests of northern South America [1]. In Ecuador, it is almost exclusively found in small cultivation sites (commonly known as *chacras*) along the western basin of the Amazon. It currently represents the most harvested and widely utilized non-food plant species by indigenous groups, like the Kichwa, Achuar and Shuar [2,3]. These ethnic groups typically use the leaves of *I. guayusa* to prepare ceremonial beverages conferring good luck for hunting and fishing and for protection against snake bites [2-5]. *Ilex guayusa* leaves are also employed for ritualistic purging, and to treat ailments like gastritis, stress and infertility [5,6]. The leaves of *I. guayusa* are rich in caffeine, terpenes, phenols and other methylxanthines, and exhibit putative anti-inflammatory, microbicidal, anti-oxidant and anti-obesity properties [4,5,7].

Historically, dried *I. guayusa* leaves were only sold in low volumes as a specialty product in local markets throughout the Ecuadorian Amazon. However, since 2009 and principally owing to the efforts of RUNA^{RM}, a social enterprise based in the USA and Ecuador, *I. guayusa* has gained commercial notoriety in international markets. It is commercialized as soluble teas and canned drinks with energizing and stimulant properties, mainly in the United States and Canada. New export markets have thus generated an increase in the number of *I. guayusa* producers in the Ecuadorian Amazon, from approximately 300 in 2011, to over 1,600 in 2013 [8]. Certainly, the growth in popularity of *I. guayusa* can bring socio-economic change to the region, but further expansion in its commercial production must be carefully evaluated as the species is native to a fragile and highly biodiverse ecosystem, vulnerable to the detrimental effects of intensive monocropping [8].

Despite its rich ethnobotanic relevance and rising commercial prospects, the biology, cultivation history and genetics of *Ilex guayusa* are poorly understood. This research presents a first assessment of the genetic diversity of *I. guayusa* in the Ecuadorian Amazon using Inter-Simple Sequence Repeat (ISSR) markers. This preliminary survey of the degree and geographic distribution of the genetic diversity of the species is necessary for establishment of diversity conservation strategies, design of plant improvement programs and generation of sustainable-agroforestry systems that best utilize plant genetic resources in vulnerable ecosystems.

MATERIALS AND METHODS

Plant material and DNA isolation

RUNA's technicians, in collaboration with students from USFQ, collected leaf samples from 157 *Ilex guayusa* individuals, from small cultivation sites listed in their database of *I. guayusa* leaf providers. The sites were distributed across the provinces of Sucumbíos (1), Napo

(91), Orellana (7), Pastaza (35), Morona Santiago (11) and Zamora Chinchipe (12); all located in the Ecuadorian Amazon (Fig 1). Collected samples were georeferenced using a Garmin E-Trex Legend HCx GPS system (Garmin International Inc., USA). For every individual, young and healthy leaves were collected from the lower branches of trees and preserved at -20°C. Genomic DNA was isolated from leaf samples using the CTAB procedure described by Kieleczawa [9]. Following extraction, DNA quality and concentration were assessed using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, USA). DNA samples were diluted to a final concentration of 20 ng/μl.

PCR amplification

Nine anchored ISSR primers were used for the molecular characterization of *Ilex guayusa* (Table 1). These primers belong to the universal UBC ISSR primer set (University of British Columbia, Canada) and had been successfully employed by Zhou [10] for the genetic characterization of diverse *Ilex* species. Genomic DNA (40 ng) was PCR amplified in a 20 μl reaction containing 50 mM KCl, 20 mM TrisHCl (pH 8.4), 1 μg/μl BSA, 2.0 mM of MgCl₂, 0.16 mM Primer Mix, 0.5 mM dNTPs, and 2 units Taq Polymerase (Life Technologies, Carlsbad, California). PCR amplification conditions consisted of an initial denaturation at 94°C for 4 min; followed by 40 cycles of a 1 min denaturation at 94°C, annealing of 50 sec at 52°C, an extension at 72°C for 1 min; and a final extension period of 7 min at 72°C. All reactions were performed in a T-Personal Series Thermocycler (Biometra, Goettingen, Germany). Amplified products were separated and visualized via electrophoresis in 1.75% w/v agarose gels, run at 90 V for 2 hours, and stained with SYBR Safe DNA gel stain (Life Technologies, Carlsbad, California). Gel band sizes were estimated using a 1 Kb DNA ladder (Life Technologies, Carlsbad, California) and band scoring was performed visually based on band-size differentiation. All bands were scored as presence/absence markers, and a binary raw-data matrix, where the presence of a band was encoded as one (1) and the absence of a band as zero (0), was generated for all gel banding patterns.

Data analysis

For all identified loci expected heterozygosity (H_e) was determined using the GenAlEx 6.501 software package [11]. Accordingly, within- and between-population estimates of molecular variance (AMOVA) and Nei genetic diversity indices were obtained using GenAlEx 6.501; assuming individual provinces as independent genetic groups, and excluding Sucumbíos because only one sample was available for this province. Principal coordinates analysis (PCoA) was performed with DARwin 5.0.15 using the DICE genetic dissimilarity coefficient [12].

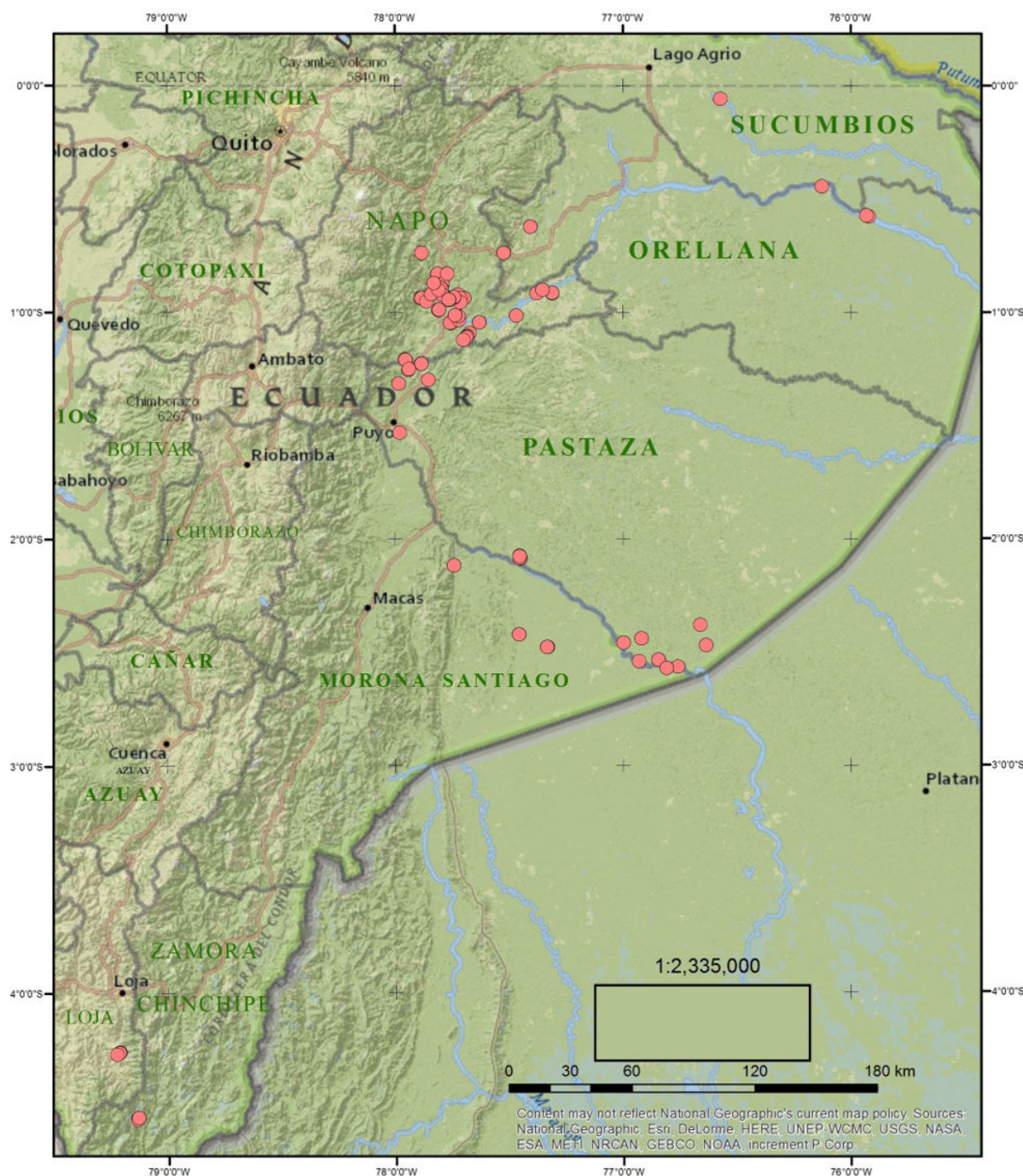


Figure 1: Location of the 157 *I. guayusa* individuals collected marked as pink circles.

RESULTS

Collectively, all evaluated ISSR markers yielded a total 91 polymorphic bands across the complete sample set (157 individuals). The total number of polymorphic loci scored per primer ranged from 3 (UBC-860) to 16 (UBC-857), with an average of 10.1 loci per primer. Of the 91 bands analyzed, 73 polymorphic loci occurred in low frequencies ($<0.5\%$) across the sample-set, but none were exclusive to any given province or genotype group. The expected heterozygosity estimate for *Ilex*

guayusa ($H_e = 0.19$) revealed a low degree of genetic diversity for the collected germplasm. Accordingly, pairwise Nei genetic distance indices implied a reduced level of genetic variability for this species (Table 2). The greatest degree of genetic differentiation was detected between individuals from Napo and Zamora Chinchipe ($D_s = 0.086$); the two most geographically distant provinces. The lowest genetic distance was detected between individuals from the neighboring provinces of Napo and Orellana ($D_s = 0.013$). While a Mantel test did not

Table 1: Information of nine ISSR anchored primers used for the molecular characterization of 157 *Ilex guayusa* individuals from the Ecuadorian Amazon.

Primer code	Primer sequence	Number of amplified bands	Number of polymorphic bands	Polymorphism percentage
UBC-815	(CT)8G	11	10	90.91
UBC-824	(TC)8G	7	6	85.71
UBC-825	(AC)8T	15	13	86.67
UBC-826	(AC)8C	12	12	100
UBC-827	(AC)8G	15	14	93.33
UBC-840	(GA)8YT	11	10	90.91
UBC-846	(CA)8RT	11	7	63.64
UBC-857	(AC)8YG	18	16	88.89
UBC-860	(TG)8RA	6	3	50
Total		106	91	85.85

Table 2: Nei genetic distance matrix comparing *Ilex guayusa* individuals from different provinces in the Ecuadorian Amazon. Sucumbíos was excluded from the analysis as only one sample was available for this province.

Napo	Orellana	Pastaza	Morona Santiago	Zamora Chinchipe
0				
0.013	0			
0.04	0.032	0		
0.04	0.023	0.025	0	
0.086	0.075	0.044	0.027	0

reveal a significant association between genetic and geographic distances ($r=0.060$; $p=0.037$), geographically distant provinces generally displayed a higher degree of genetic divergence.

Analysis of molecular variance (AMOVA) demonstrated that the highest percentage (82%) of genetic variation for *Ilex guayusa* was found within groups (individuals belonging to the same province), relative to among groups (18%). This factor indicates a low degree of population differentiation. Nevertheless, Principle Coordinates Analysis (PCoA) (Fig 2) could resolve collected germplasm into three robust genetic clusters as determined by its first two coordinates, each respectively accounting for 23% and 11% of observed variation. Cluster A included individuals from the most-northern areas of the provinces of Napo and Orellana, as well as the only individual from the province of Sucumbíos. Cluster B included individuals from central and northern quadrants of Napo, and individuals from the province of Orellana that were geographically proximal to Napo's eastern provincial border. Finally, Cluster C comprised all individuals from the southern quadrants of Napo, and individuals from the southern Amazonian provinces of Pastaza, Morona-Santiago and Zamora Chinchipe.

DISCUSSION

The main objective of this study was to assess the level of genetic diversity of *Ilex guayusa*, a species of great ethnobotanic and commercial importance for indigenous communities of the Ecuadorian Amazon [2]. Overall, expected heterozygosity ($H_e = 0.19$) revealed a reduced level of genetic variability for the species. For comparison, using five isoenzyme markers, Winge *et al.* [13] detected a comparatively higher degree of heterozygosity ($H_e = 0.50$) for *Ilex paraguariensis*, a close relative of *I. guayusa*. The RAPD-based characterization of four natural populations of *I. paraguariensis* from southern Brazil revealed that the average (Nei) genetic distance between individuals within a population was 0.392 [14], whereas the maximum degree of genetic differentiation between *I. guayusa* individuals analyzed in this study was 0.086 (Table 2). At first hand, the low level of genetic variability observed for Ecuadorian *I. guayusa* appears contradictory, especially when considering the extensive number of polymorphic loci (91) detected across the evaluated sample-set. However, it is important to highlight that approximately 80% of all detected polymorphisms occurred in low frequencies ($<5\%$), and the vast majority of these did not exhibit correlative patterns of allelic fixation for specific genotypes or genotype groups (i.e. individuals from specific provinces).

The low degree of genetic diversity found for *Ilex guayusa* could most likely be ascribed to the fact that all col-

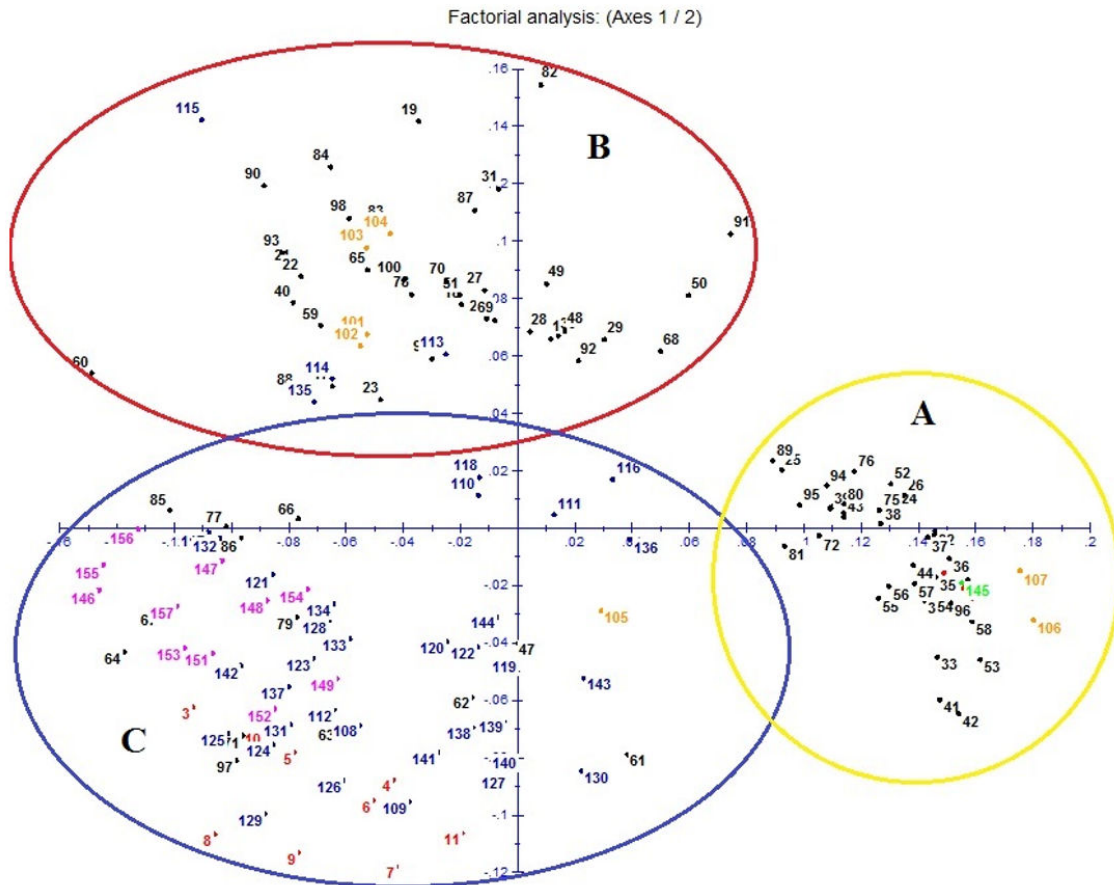


Figure 2: PCoA of the 157 *Ilex guayusa* individuals. Each number corresponds to an individual, from one of the six provinces of Ecuadorian Amazon, grouped in clusters A, B and C: Sucumbíos (green), Napo (black), Orellana (orange), Pastaza (blue), Morona-Santiago (red) and Zamora-Chinchi (purple).

lected germplasm originates from small cultivated plots. In the Ecuadorian Amazon, indigenous groups cultivate *I. guayusa* exclusively via clonal propagation through stem cuttings, a cultural activity that has probably homogenized the species' genetic pool across its geographic range of cultivation [16-18]. Historically, *I. guayusa* has existed in close relationship with Amazonian ethnic and cultural processes, and the species' sampled geographic distribution likely reflects the migration patterns of indigenous groups [15]. Moreover, no reports exist of wild *I. guayusa* populations, and it is therefore impractical to extrapolate on whether the species displays a generalized low degree of genetic diversity. The results of this study also imply that in the Ecuadorian Amazon, cultivated *I. guayusa* originates from a specific and reduced gene pool. Partitioning of genetic diversity (AMOVA) indicated that 82% of the variation observed for this species occurred within populations (provinces), and only 18% between populations. Natural populations of *I. paraguariensis* have also shown a reduced degree of genetic differentiation, principally accounted to the species' outcrossing nature [14]. In outcrossing perennial tree species, natural populations generally exhibit a high degree of allelic exchange via gene flow; a factor which limits genetic divergence between

populations [19]. Yet, because samples analyzed in this study come from cultivated plots established through clonal propagation, it seems probable that the high degree of genetic homogeneity observed for *I. guayusa* results from the widespread propagation of a selection of homogenous genotypes with desirable characteristics and established for cultivation prior to the anthropogenic-driven dispersion of the species across the Amazon basin [5].

Remarkably, while genetic differentiation between established groups was limited, a subtle genetic structure could be established for Ecuadorian *Ilex guayusa*. Principle Coordinates Analysis (PCoA) could resolve collected germplasm into three distinct groups. All clusters included a high proportion of individuals from the province of Napo, which was over-represented in this study. However, each cluster was distinctly and respectively characterized by the presence of individuals from the remaining northern, central and southern provinces of the Ecuadorian Amazon (Fig 2). For decades, Napo has represented the center of economic activity in the Ecuadorian Amazon, and around 50% of its population is composed of migrants from neighboring provinces. It is probable that some of these migrants brought specific

variants of *I. guayusa* for cultivation [20]. Incidentally, PCoA clustering tended to group individuals from Napo in a directional gradient, from north to south, possibly highlighting the geographically structured introduction of novel *I. guayusa* alleles from distinct, yet proximal geographic locations in neighboring provinces. Certainly, the inclusion of a higher proportion of individuals from underrepresented provinces (*i.e.* Sucumbíos), as well as wild populations (if these should exist), would help us better resolve the geographic distribution of *I. guayusa* genetic diversity along the Amazon basin of Ecuador, and formulate stronger conclusions regarding its cultivation history in the region. This information will be crucial for the establishment of adequate conservation strategies aimed at preserving and promoting the use of unique sources of genetic variation which can increase the productivity and environmental resilience of cultivated orchards under sustainable agro-forestry systems. Expectantly, the latter will enable for a controlled and sustainable expansion of *I. guayusa* cultivation in the Ecuadorian Amazon which can meet rapidly growing commercial demands, and reduce the need for intensive mono-cropping systems and their associated detrimental effects on vulnerable ecosystems.

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AUTHORS CONTRIBUTIONS

Andres Torres supervised research and wrote the paper; Juan Mosquera performed the experiments, analyzed the data and contributed in writing the paper; Viviana Jaramillo supervised research and helped in conducting experiments and data analysis; Venancio Arahana helped to design the experiments and data analysis; María de Lourdes Torres conceived and supervised the research, obtained the funds, and performed critical reading of the manuscript.

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